

## Phosphodiesterase 4 Inhibitors Augment Levels of Glucocorticoid Receptor in B Cell Chronic Lymphocytic Leukemia but Not in Normal Circulating Hematopoietic Cells

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**Abstract** Type 4 cyclic AMP (cAMP) phosphodiesterase (PDE4) inhibitors, a class of compounds in clinical development that activate cAMP-mediated signaling by inhibiting cAMP catabolism, offer a feasible means by which to potentiate glucocorticoid-mediated apoptosis in lymphoid malignancies such as B-cell chronic lymphocytic leukemia (B-CLL). In this study, we show that PDE4 inhibitors up-regulate glucocorticoid receptor (GR $\alpha$ ) transcript levels in B-CLL cells but not T-CLL cells or Sezary cells or normal circulating T cells, B cells, monocytes, or neutrophils. Because GR $\alpha$  transcript half-life does not vary in CLL cells treated with the prototypic PDE4 inhibitor rolipram, the 4-fold increase in GR $\alpha$  mRNA levels observed within 4 h of rolipram treatment seems to result from an increase in GR $\alpha$  transcription. Rolipram treatment increases levels of transcripts derived from the 1A3 promoter to a greater extent than the 1B promoter. Treatment of B-CLL cells with two other PDE4 inhibitors currently in clinical development also augments GR transcript levels and glucocorticoid-mediated apoptosis. Washout studies show that simultaneous treatment with both drug classes irreversibly augments apoptosis over the same time frame that GR up-regulation occurs. Although treatment of B-CLL cells with glucocorticoids reduces basal GR $\alpha$  transcript levels in a dose-related manner, cotreatment with rolipram maintained GR $\alpha$  transcript levels above baseline. Our results suggest that as a result of their unusual sensitivity to PDE4 inhibitor-mediated up-regulation of GR $\alpha$  expression, treatment of B-CLL patients with combined PDE4 inhibitor/glucocorticoid therapy may be of therapeutic benefit in this disease.

Glucocorticoids are an important component of standard therapy for several lymphoid malignancies, including multiple myeloma, acute lymphoblastic leukemia, and diffuse large B-cell lymphoma. As early studies in patients with B-cell chronic lymphocytic leukemia (B-CLL) showed that addition of prednisone to chlorambucil augmented response rate but not median survival, glucocorticoids are not generally a standard component of initial therapy for patients with B-CLL (1, 2). Nonetheless, two studies of high-dose glucocorticoid therapy have suggested that glucocorticoids can be of clinical benefit to a subset of patients with treatment-refractory B-CLL (3, 4).

Despite frequent responses to glucocorticoid treatment, monotherapy with glucocorticoids is not curative in any lymphoid malignancy, but the mechanisms underlying clinical glucocorticoid resistance remain controversial. Structural alterations in the glucocorticoid receptor (GR) are commonly

identified in lymphoid cell lines that have been selected for glucocorticoid resistance by prolonged culture in dexamethasone, but comparable alterations in primary malignant lymphoid cells have been only infrequently reported (5–9). A detailed analysis of treated B-CLL patients failed to identify abnormalities in either the DNA- or steroid-binding domains of leukemic GRs (10). Nonstructural modifications of glucocorticoid signaling pathways are likely to be important in clinical glucocorticoid resistance, and efforts to identify and reverse these modifications may be therapeutically useful (8, 9). Several clinical studies in patients with acute and chronic lymphoid leukemias have reported a correlation between low leukemic cell GR $\alpha$  expression levels and poor response to treatment (11–13). However, numerous exceptions to such correlative studies have also been reported, leading to the suggestion that clinical resistance to glucocorticoids may also result from unrelated downstream signaling alterations (reviewed in ref. 14).

Cyclic AMP (cAMP)-mediated signaling can favorably alter the apoptotic response to glucocorticoids in specific lymphoid subsets, although the precise molecular explanation for this relationship remains unclear. Seminal early work done by S. Bourgeois et al. showed that isolation of WEHI-7 cells, a murine T-cell lymphoma line, that were resistant to cAMP-mediated apoptosis due to alterations in protein kinase A (PKA) made the subsequent development of spontaneous glucocorticoid-resistant cells occur at higher frequencies ( $10^{-7}$ ) than in wild-type cells ( $<10^{-10}$ , ref. 15). Gruol and Altschmid subsequently determined that RU486, ordinarily a GR antagonist for

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glucocorticoids-induced lymphoid cytotoxicity, becomes an agonist in the setting of cotreatment with a cAMP analogue (16). Conversely, McConkey et al. reported that GR-deficient ICR 27 cells, a variant of the CEM T-cell lymphoma line, are insensitive to cAMP-induced apoptosis. Transfection of ICR 27 cells with the GR restored sensitivity to cAMP-mediated apoptosis (17). Finally, the catalytic subunit of PKA has been shown to associate with the GR (18).

One critical factor that regulates lymphoid sensitivity to glucocorticoids is the level of GR expression. Gruol et al. determined that treatment of Wehi-7 cells with cAMP analogues raised GR transcript and protein levels (19). Several mechanisms have been proposed to explain why GR transcript levels increase following treatment of specific cell subsets with agents that augment cAMP signaling. In studies of rat hepatoma cells, Dong et al. (20) reported that treatment with 8-bromo-cAMP increases GR mRNA half-life from 4 to 10 h. Because treatment of such cell cultures with inhibitors of protein or mRNA synthesis had no effect on the ability of 8-bromo-cAMP to increase GR transcript levels, Dong et al. argue that a principal mechanism by which cAMP signaling augments GR transcript levels must be through GR mRNA stabilization. In contrast, using transfection of GR promoter luciferase constructs into HeLa cells, Penuelas et al. (21) determined that treatment with the adenylate cyclase activator forskolin doubled the transcriptional activity of the human GR promoter. After mapping and testing five putative cAMP response element (CRE)-binding sites, the authors showed loss of forskolin inducibility in promoter constructs shorter than 1 kb and the presence of a CRE element that bound CRE *in vitro* in shift assays. Thus, it seems that in some cell lineages, cAMP-induced augmentation of GR transcript levels is due to augmented transcription rather than mRNA stabilization.

Type 4 cAMP phosphodiesterase (PDE4) inhibitors offer a therapeutically plausible means by which to take advantage of the phenomenon of cAMP-mediated augmentation of glucocorticoid sensitivity in malignant lymphoid cells. The PDE4 family plays a key role in catabolizing cAMP in a variety of human hematopoietic cells, and PDE4 inhibitors are in late-stage clinical studies for a variety of inflammatory illnesses, including asthma and chronic obstructive disease (22). In prior work, we have determined that inhibition of PDE4, in the absence of the addition of exogenous adenylate cyclase activators such as forskolin or  $\beta$ -adrenergic agonists, increases cAMP levels, activates PKA as judged by cAMP-response element binding protein (CREB) phosphorylation, and induces apoptosis in primary B-CLL cells, albeit in well less than 100% of cells (23). Treatment with the prototypic PDE4 inhibitor rolipram induces mitochondrial release of cytochrome *c*, activation of caspase-9 and caspase-3, and cleavage of poly(ADP-ribose) polymerase in CLL cells (24). PDE4 inhibitors also activate Rap1 in B-CLL cells due to cAMP-mediated activation of the Rap1 GDP exchange factor EPAC1, but EPAC activation seems to be antiapoptotic (25). PDE4 inhibitors thus induce both PKA-mediated proapoptotic and EPAC-mediated antiapoptotic signaling pathways in B-CLL cells, with the PKA-mediated proapoptotic pathway having a dominant effect.

PDE4 inhibitors such as rolipram augment hydrocortisone- or dexamethasone-induced apoptosis in primary B-CLL cells, as well as transactivation of glucocorticoid response element (GRE)-containing reporter constructs (26). Both of these

effects are reversed by the type I PKA antagonist Rp-8Br-cAMPS. The specific mechanism or mechanisms by which PDE4 inhibitors increase glucocorticoid sensitivity in B-CLL cells remain unknown. In this study, we sought to determine whether PDE4 inhibitors alter leukemic expression of GR. We find that PDE4 inhibitors augment expression of GR at a transcriptional level, and that among human primary hematopoietic cells, this effect is quite specific to B-CLL.

## Materials and Methods

**Materials.** The following reagents were obtained from commercial sources: Rolipram (Biomol), forskolin (Sigma), actinomycin D (Calbiochem), and Rp-8-Br-cAMPS (Biolog). Cilomilast and rolipram were obtained from Memory Pharmaceuticals.

**Cell culture and isolation.** Blood samples were obtained in heparinized tubes with Institutional Review Board-approved consent from flow cytometry-confirmed B-CLL patients that were either untreated or for whom at least 1 month had elapsed since chemotherapy. Patients with active infections or other serious medical conditions were not included in this study. Patients with WBC counts of  $<15,000/\mu\text{L}$  by automated analysis were excluded from this study. Whole blood was layered on Ficoll-Histopaque (Sigma) and peripheral blood mononuclear cells (PBMCs) isolated after centrifugation. PBMCs were washed and resuspended at  $1 \times 10^7$  cells/mL in complete media [RPMI 1640 (Mediatech) supplemented with 10% fetal bovine serum (Sigma), 20 mmol/L L-glutamine, 100 IU/mL penicillin, and 100  $\mu\text{g}/\text{mL}$  streptomycin (Mediatech)]. PBMCs were found to contain  $>90\%$  B-CLL by fluorescence-activated cell sorting (FACS) without additional purification. Normal B cells, T cells, and monocytes were obtained from anonymous healthy donors (New York Biologics) and isolated via antibody-coupled magnetic bead-negative depletion per the manufacturer's protocol (Miltenyi) from PBMC. Polymorphonuclear cells (PMNs) were obtained by erythrocyte depletion of whole blood via dextran sedimentation, followed by removal of PBMCs using Ficoll separation. With the exception of PMNs, which were used immediately after purification, all other primary normal and malignant cell populations were rested overnight at  $37^\circ\text{C}$  before use.

**Western blot analysis.** Following cell culture, cells were collected by centrifugation ( $400 \times g$ ; 10 min), washed once with PBS, and lysed in ice-cold 10 mmol/L HEPES-NaOH buffer (pH, 7.4) containing 1% Triton X-100, 10% glycerol, 25 mmol/L  $\beta$ -glycerophosphate, 100 mmol/L NaCl, 2 mmol/L EDTA, 2 mmol/L EGTA, 1 mmol/L DTT, 1 mmol/L Vanadate, 1 mmol/L phenylmethylsulfonyl fluoride, and 1 mmol/L benzamide. Cell lysates were transferred to 1.5-mL tubes and centrifuged at 14,000 rpm for 30 min ( $5^\circ\text{C}$ ) in a microcentrifuge to clarify samples of insoluble cellular debris. Concentrations of soluble proteins in samples of clarified supernatants were determined using Bradford assays. Samples were heat denatured at  $100^\circ\text{C}$  for 5 min in protein-denaturing sample buffer. Levels of GR protein expression were examined in 50- $\mu\text{g}$  aliquots of denatured protein samples that were subjected to electrophoretic separation through 8% SDS-polyacrylamide gels followed by electrotransfer onto Immobilon-P membrane (Millipore) in 10 mmol/L 3-(cyclohexylamino)-1-propanesulfonic acid buffer (pH, 11) containing 10% methanol. Primary GR antibodies (Santa Cruz Biotechnology, sc-1002) and secondary goat anti-rabbit immunoglobulin G (IgG) conjugated to horseradish peroxidase (HRP, Santa Cruz Biotechnology, sc-2004) were diluted at 1:500 and 1:5,000, respectively, in TBS [20 mmol/L Tris-HCl (pH, 7.6); 137 mmol/L NaCl, 0.1% Triton X-100] containing 5% nonfat milk (w/v) to immunoblot proteins on Western blot membranes. Immunocomplexes with HRP activity on membranes were developed using enhanced chemiluminescence reagent (Pierce) as substrate and visualized by exposure to an X-ray film. Membranes were blotted with primary monoclonal anti- $\alpha$ -tubulin (Sigma, T5168) to control for equal loading of protein samples on gels and transfer onto membranes.

**Real-time PCR for GR exon 1A3-2, 1B-2, 1C-2, 8-9 $\alpha$ , and 8-9 $\beta$  transcripts.** Cells were plated at a concentration of  $1 \times 10^5$  cells/mL in complete media with or without drug treatment as indicated and incubated at 37°C for various times as indicated. Total RNA was obtained using the RiboPure isolation kit (Ambion) per the manufacturer's protocol. Reverse transcription of 2 to 5  $\mu$ g of total RNA was carried out with the Superscript III reverse transcription kit (Invitrogen) primed with random hexamers. An assay for the GR exon 8-9 $\alpha$  transcript was devised using primer3 software.<sup>3</sup> The forward and reverse flanking oligonucleotide primers were 5'-ACGGATGTGCAAGGCAAG-3' and 5'-TGATGTGCTGATTCAGCTA-3', respectively. The FAM/TAMRA TaqMan probe located in exon 8 was 5'-TGACAGGCAAGCTGGCAGGCG-3'. Each reaction contained 900 nmol/L forward and reverse primer, 50 nmol/L probe, 1 $\times$  Universal PCR Master Mix (Applied Biosystems), and 2  $\mu$ L of cDNA diluted 1:50. Flanking primers and internal FAM/TAMRA-labeled probe oligonucleotides and reaction conditions for the splice sites at GR exons 1A3-2, 1B-2, and 1C-2, as well as GR exon 8-9 $\beta$  were those reported by Pedersen and Vedeckis, with the exception that the TaqMan probe for the exon 1A3-2 site was (27) 5'-TCATCAATATCAACTCTTCGACACACTTAATGA-3' and the reverse primer for the exon 8-9 $\beta$  splice site was 5'-TCTGCAATGCTGCTTCTGTTTAA-3' (28). cDNA was diluted 1:50 for measurement of exon 1A3-2, 1B-2, and 1C-2 and diluted 1:5 for measurement of exon 8-9 $\beta$ . Predeveloped TaqMan assay reagents for measuring 18S rRNA (Applied Biosystems) was used for normalization. Real-time PCR was carried out on an MX300P instrument (Stratagene). Transcript abundance relative to control samples was calculated using the  $2^{-\Delta\Delta C_T}$  method. We established that the slopes of the curves for the amplification of GR $\alpha$  and rRNA did not vary by more than 10%. All oligonucleotides were purchased from IDT.

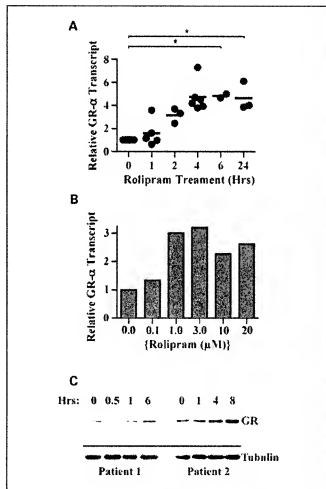
**Apoptosis assays.** One million cells were incubated in duplicate in 48-well plates with or without drug treatment as indicated for 48 h in 1 mL of complete media. Cells were transferred to polypropylene Falcon FACS tubes and incubated for 10 min at 37°C with Hoechst 33342 (Molecular Probes) at a final concentration of 0.25  $\mu$ g/mL. Cells were then stored on ice until analysis on a MoFlo cytometer using a 450-nm bandpass filter. In some cases, apoptosis was detected as membrane depolarization with dihexyloxycarbocyanine (DiOC<sub>6</sub>(3)) from Molecular Probes at a concentration of 400 nmol/L. DiOC<sub>6</sub>(3)-stained samples were incubated for 30 min at 37°C and stored on ice until analysis on a FACScan (Becton Dickinson). Results obtained from Hoechst 33342 and DiOC<sub>6</sub>(3) staining were validated with Annexin V-propidium iodide (PI) staining per the manufacturer's protocol (BD). FACS data were analyzed using FlowJo software (Tree Star Inc.) by gating for the apoptotic population. The level of apoptosis detected in B-CLL cultures did not differ by more than 10% when measured by Hoechst 33342 or DiOC<sub>6</sub>(3).

**Statistical analysis.** Statistical analysis and plotting were done using Prism version 4 (GraphPad Software) and SPSS version 12 (SPSS Inc.). The significance of the main and interaction effects was determined via repeated-measures ANOVA tests with significance of subsequent pairwise comparisons via Bonferroni post hoc tests. In some cases multivariate ANOVA (MANOVA) were used as indicated when the data violated the sphericity assumption for ANOVA.

## Results

**Rolipram augments GR $\alpha$  transcript and protein levels in B-CLL cells in a time- and dose-dependent manner.** Given prior reports that cAMP analogues augment GR levels in a subset of cell types, we used comparative quantification real-time reverse transcription-PCR (RT-PCR) to determine whether treatment of B-CLL cells with a PDE4 inhibitor altered expression of GR $\alpha$

transcript. In leukemic cells from eight patients, treatment of B-CLL cells with the PDE4 inhibitor rolipram (20  $\mu$ M/L) augmented GR $\alpha$  transcript levels in a time- and dose-dependent manner. The effect of rolipram exposure time on GR $\alpha$  transcript level was found to be significant by ANOVA ( $P = 0.017$ ). GR $\alpha$  transcript levels rose significantly over the first 6 h to a mean of  $4.8 \pm 0.2$ -fold above baseline ( $P = 0.028$ ) and maintained such a 4-fold increase for at least 24 h (Fig. 1A). Although comparable augmentation of GR transcript levels was observed at rolipram doses ranging from 1 to 20  $\mu$ M/L, significant augmentation was not observed at 0.1  $\mu$ M/L rolipram, a concentration at or below the EC<sub>50</sub> of rolipram for the



**Fig. 1.** GR $\alpha$  expression is up-regulated in B-CLL cells following treatment with the PDE4 inhibitor rolipram. **A**, B-CLL cells were treated for the indicated lengths of time with rolipram (20  $\mu$ M/L), followed by RNA isolation, cDNA synthesis, and real-time PCR for GR $\alpha$  using oligonucleotides that spanned exons 8 and 9 $\alpha$ . Each point represents the fold increase in GR $\alpha$  transcript levels of an individual patient sample relative to the same patient's CLL cells treated with vehicle (DMSO) alone. The mean fold increase in transcript level is denoted with a horizontal line. \*, significant main effect for time at  $P < 0.05$  (ANOVA). **B**, B-CLL cells from an individual patient were treated for 4 h with DMSO or rolipram at the indicated dosage ( $\mu$ M/L), followed by RNA isolation and real-time RT-PCR for GR $\alpha$  transcript levels relative to vehicle (DMSO) control. The data are representative of one of two similar experiments. **C**, B-CLL cells were treated with DMSO alone (0 h time point) or rolipram (20  $\mu$ M/L) for the indicated amount of time, followed by lysis, protein quantification, and immunoblot analysis for GR $\alpha$  protein expression (GR).  $\alpha$ -Tubulin was also assessed by immunoblot analysis as an internal loading control. Results from two patients are shown and are representative of four patients tested.

<sup>3</sup> Available at <http://trodz.wi.mit.edu/cgi-bin/primer3/primer3.www.cgi>.

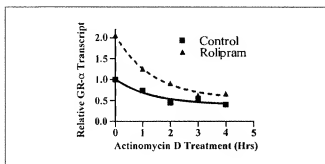


Fig. 2. PDE4 inhibitor-induced GR $\alpha$  transcript up-regulation is not due to altered transcript half-life. B-CLL cells were treated for 4 h with vehicle control alone (DMSO) or rolipram (20  $\mu$ M/L) followed by treatment with the RNA polymerase inhibitor actinomycin D (10  $\mu$ M/L) for the indicated period of time. After RNA isolation and cDNA synthesis, samples were analyzed for relative GR $\alpha$  transcript levels by real-time PCR. All GR transcript levels were normalized to that observed in DMSO-treated cells at time 0. A nonlinear curve fit for one phase exponential decay revealed no significant difference in the rates of reduction in GR $\alpha$  transcript levels ( $P = 0.88$ ). The experiment is representative of two experiments done.

inhibition of tumor necrosis factor secretion (Fig. 1B; ref. 29). Addition of the adenylate cyclase stimulator forskolin did not significantly augment GR $\alpha$  transcript in B-CLL cells, either when used alone or in combination with rolipram, a finding in keeping with prior studies demonstrating that rolipram activates PKA in B-CLL in the absence of exogenous adenylate cyclase activation (data not shown). Western blot analysis of rolipram-treated B-CLL cells from four patients showed that PDE4-inhibitor-induced GR transcript up-regulation was associated with an increase in GR protein at 4 to 6 h (Fig. 1C).

cAMP-mediated augmentation of GR transcript levels has been variably attributed to increased GR half-life (in rat hepatoma cells) or GR transcription (in HeLa cells; refs. 20, 21). To establish whether the increased levels of GR transcript observed in rolipram-treated B-CLL cells were the result of altered transcript half-life, we treated B-CLL cells with vehicle alone (DMSO) or rolipram (20  $\mu$ M/L) for 4 h, followed by treatment with the RNA polymerase inhibitor actinomycin D (10  $\mu$ M/L) for varying periods of time. Assessment of GR $\alpha$  transcript levels following such actinomycin D treatment revealed that the half-life of GR $\alpha$  transcript was not altered by rolipram treatment ( $P = 0.88$ ; Fig. 2), suggesting that in B-CLL cells, cAMP-mediated augmentation of GR $\alpha$  transcript occurs by a transcriptional mechanism.

**Rolipram-mediated GR $\alpha$  transcript up-regulation is not observed in a range of normal hematopoietic cell types.** To establish the specificity of PDE4 inhibitor-mediated GR transcript up-regulation, we carried out real-time RT-PCR analyses in a range of normal hematopoietic cells. Rolipram treatment did not augment GR $\alpha$  transcript levels in unpurified human mononuclear cells or in purified populations of human T cells, B cells, neutrophils, and monocytes (Fig. 3). In the absence of basal adenylate cyclase activity, PDE4 inhibitors may be relatively ineffective in activating cAMP-mediated signal transduction. However, forskolin (40  $\mu$ M/L), either alone or in combination with rolipram, did not induce GR transcript up-regulation in these normal hematopoietic cell populations (data not shown). Because it is possible that a PDE family other than PDE4 might regulate GR levels in these cell populations, we examined whether the addition of IBMX (50  $\mu$ M/L), a competitive inhibitor of 9 of the 11 currently known PDE

families, led to an increase in GR transcript. No augmentation of GR transcript levels was observed in unpurified human mononuclear cells (a predominantly T-cell preparation) or in purified populations of human monocytes (data not shown). To establish whether the observed effect of PDE4 inhibitors on B-CLL GR $\alpha$  transcript levels was simply a function of lymphoid transformation, we examined primary leukemic cells from a patient with T-CLL and a patient with Sezary syndrome. In neither case was rolipram-induced augmentation of GR $\alpha$  transcript levels observed (Fig. 3).

**Roflumilast and cilomilast augment glucocorticoid-mediated apoptosis and GR $\alpha$  transcript levels.** To determine whether the alterations in GR $\alpha$  transcript observed following rolipram treatment of B-CLL cells are shared by other structurally distinct PDE4 inhibitors, we examined cilomilast and roflumilast, two PDE4 inhibitors that have been used in clinical trials testing the activity of PDE4 inhibitors in asthma and chronic obstructive pulmonary disease (Fig. 4A; refs. 30, 31). Consistent with the hypothesis that PDE4 is in fact the rolipram target that results in augmented GR $\alpha$  transcript levels, cilomilast and roflumilast increased GR $\alpha$  transcript levels ( $P = 0.012$ ; ANOVA) in B-CLL cells (Fig. 4B). As previously observed with rolipram, both cilomilast (50  $\mu$ M/L) and roflumilast (1.0  $\mu$ M/L) augmented the efficacy with which glucocorticoids induce apoptosis in B-CLL (Fig. 4C). In pooled data from 10 B-CLL patients, combining PDE4 inhibitor and glucocorticoid treatment significantly augmented apoptosis relative to either agent alone ( $P < 0.01$ ; MANOVA). Despite this statistically significant effect, however, it is important to point out that among these 10 leukemic cell samples, several did not in fact show PDE4 inhibitor-mediated augmentation of glucocorticoid apoptosis. Such heterogeneity is similar to results we previously obtained in studies with rolipram. One patient whose leukemic cells were very sensitive to glucocorticoid-mediated apoptosis showed no further augmentation with the addition of cilomilast or roflumilast. Another leukemic cell sample that had relatively high basal apoptosis (39%) showed little or no sensitivity to any of the drug treatments. It is possible that this

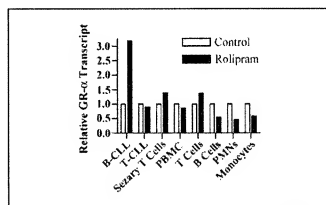


Fig. 3. Among circulating hematopoietic lineage cells tested, PDE4 inhibitor-induced augmentation of GR $\alpha$  transcript levels is specific to B-CLL. B-CLL cells, T-CLL cells, Sezary cells, human PBMCs, T cells, B cells, neutrophils (PMNs) or monocytes were incubated for 4 h in vehicle control alone (DMSO) or rolipram (20  $\mu$ M/L) as indicated. RNA was isolated, cDNA synthesized, and levels of GR $\alpha$  transcript relative to vehicle-treated cells of each cell type was assessed by real-time RT-PCR. The data are representative of at least two samples tested for all cell populations except T-CLL, where only one patient sample was available.

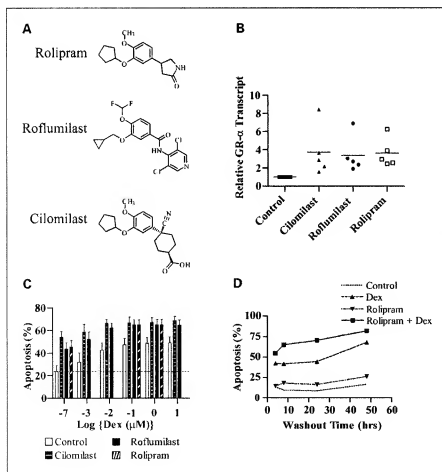
heterogeneity in apoptotic responses to combined glucocorticoid/PDE4 inhibitor treatment is due to genetic heterogeneity of the leukemias in this patient population.

**The synergistic apoptotic effects of combined PDE4 inhibitor/glucocorticoid treatment can be observed following drug treatment for as little as 2 h.** If the potential therapeutic benefit of combined PDE4 inhibitor/glucocorticoid therapy is to be explored clinically, it will be important to determine the length of time leukemic cells must be exposed to both agents to augment glucocorticoid-mediated apoptosis. CLL cells were treated with vehicle alone, rolipram (20  $\mu\text{mol/L}$ ), dexamethasone (1  $\mu\text{mol/L}$ ), or the combination of rolipram and dexamethasone for varying periods of time, followed by washing and completion of cell culture for 48 h. Combined rolipram/glucocorticoid treatment for as little as 2 h augmented apoptosis relative to treatment with either drug alone (Fig. 4D). Treatment for 8 h with the drug combination resulted in a level of apoptosis that approached that observed following a full 48 h of combined drug treatment. Although our studies did not address how much of the compounds remain associated with the leukemic cells after washing, these results suggest that PDE4 inhibitors potentiate glucocorticoid-mediated apoptosis in a relatively short period of time.

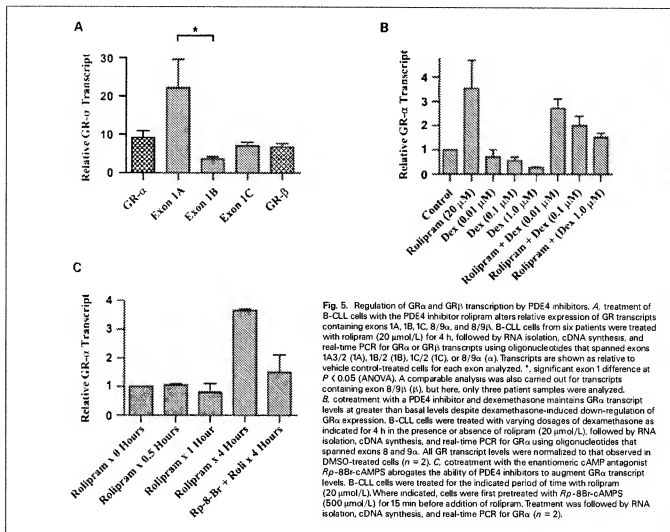
**PDE4 inhibitors variably augment different classes of GR $\alpha$  transcript.** Transcription of the GR gene is regulated by three promoters, 1A, 1B, and 1C (32). Prior studies in the human

B-cell line IM-9 have shown that under basal conditions, roughly 1%, 23%, and 76% of GR $\alpha$  transcripts are derived from promoters 1A, 1B, and 1C, respectively (28). Using previously validated real-time PCR assays that detect splicing of exons 1A3, 1B, and 1C to exon 2, we examined leukemic cells from six B-CLL patients for the effect of rolipram treatment on levels of GR $\alpha$  transcripts derived from these three promoters. As illustrated in Fig. 5A, rolipram (20  $\mu\text{mol/L}$ ) augmented GR transcripts derived from each of the three promoters: exon 1A3 ( $22.2 \pm 7.4$ -fold), exon 1B ( $3.6 \pm 0.5$ -fold), and exon 1C ( $7.1 \pm 0.9$ -fold). The up-regulation observed for transcripts containing exon 1A3 was significantly higher than that observed for transcripts containing exon 1B ( $3.6 \pm 0.5$ -fold;  $P < 0.05$ ; ANOVA).

GR $\beta$  has been reported to suppress transactivation of GR $\alpha$  by synthetic glucocorticoids, and high levels of GR $\beta$  may correlate with insensitivity to glucocorticoid-induced apoptosis (33, 34). We therefore examined GR $\beta$  regulation by PDE4 inhibitors in B-CLL. Treatment with rolipram augmented GR $\beta$  transcript levels 7-fold relative to levels observed in untreated CLL cells (Fig. 5). Basal levels of GR $\beta$  in B-CLL cells seem to be far lower than those of GR $\alpha$  because the real-time PCR threshold cycle numbers we observed for GR $\beta$  were 10 cycles greater than those for GR $\alpha$  despite comparable efficiency of amplification. These results are similar to the 1,000-fold lower GR $\beta$  level reported by Vedeckis et al. (28) using the same oligonucleotide primers in



**Fig. 4.** Effect of several structurally distinct PDE4 inhibitors on glucocorticoid-mediated apoptosis and GR $\alpha$  transcript levels. **A**, molecular structures of rolipram, roflumilast, and cilomilast. **B**, B-CLL cells were treated as indicated with vehicle control (DMSO), 50  $\mu\text{mol/L}$  cilomilast, 1.0  $\mu\text{mol/L}$  roflumilast or 20  $\mu\text{mol/L}$  rolipram for 4 h followed by RNA isolation and RT-PCR analysis for GR $\alpha$  transcript. The mean fold increase in transcript level of five patients is denoted with a horizontal line. **C**, B-CLL cells were treated for 48 h with PDE4 inhibitors alone or in the presence of increasing dosages of dexamethasone, as indicated, followed by assessment for apoptosis by FACS analysis. The PDE4 inhibitors cilomilast, roflumilast, and rolipram were added at 50, 10, and 20  $\mu\text{mol/L}$ , respectively. Columns, mean of 10 patients tested; bars, SE. **D**, B-CLL cells were treated for 0, 4, 8, 24, or 48 h with vehicle alone, rolipram (20  $\mu\text{mol/L}$ ), dexamethasone (1  $\mu\text{mol/L}$ ), or the combination of rolipram and dexamethasone. The drugs were removed by washing at the indicated time, followed by completion of culture in media alone until 48 h had elapsed.



**Fig. 5.** Regulation of GR $\alpha$  and GR $\beta$  transcription by PDE4 inhibitors. **A**, treatment of B-CLL cells with the PDE4 inhibitor rolipram alters relative expression of GR transcripts containing exons 1A, 1B, 1C, 8/9 $\alpha$ , and 8/9 $\beta$ . B-CLL cells from six patients were treated with rolipram (20  $\mu$ M/L) for 4 h, followed by RNA isolation, cDNA synthesis, and real-time PCR for GR $\alpha$  or GR $\beta$  transcripts using oligonucleotides that spanned exons 1A3/2 (1A), 1B/2 (1B), 1C/2 (1C), or 8/9 $\alpha$  ( $\alpha$ ) or 8/9 $\beta$  ( $\beta$ ). Transcripts are shown as relative to vehicle control-treated cells for each exon analyzed. \*, significant exon 1 difference at  $P < 0.05$  (ANOVA). A comparable analysis was also carried out for transcripts containing exon 8/9 $\beta$  ( $\beta$ ), but here, only three patient samples were analyzed. **B**, cotreatment with a PDE4 inhibitor and dexamethasone maintains GR $\alpha$  transcript levels at greater than basal levels despite dexamethasone-induced down-regulation of GR $\alpha$  expression. B-CLL cells were treated with varying dosages of dexamethasone as indicated for 4 h in the presence or absence of rolipram (20  $\mu$ M/L), followed by RNA isolation, cDNA synthesis, and real-time PCR for GR $\alpha$  using oligonucleotides that spanned exons 8 and 9 $\alpha$ . All GR transcript levels were normalized to that observed in DMSO-treated cells ( $n = 2$ ). **C**, cotreatment with the enantiomeric cAMP antagonist Rp-8-Br-cAMPS abrogates the ability of PDE4 inhibitors to augment GR $\alpha$  transcript levels. B-CLL cells were treated for the indicated period of time with rolipram (20  $\mu$ M/L). Where indicated, cells were first pretreated with Rp-8-Br-cAMPS (500  $\mu$ M/L) for 15 min before addition of rolipram. Treatment was followed by RNA isolation, cDNA synthesis, and real-time PCR for GR $\alpha$  ( $n = 2$ ).

a quantitative real-time RT-PCR study of basal and glucocorticoid-treated GR $\alpha$  and GR $\beta$  transcript levels in the EBV-transformed B-cell line IM-9.

**PDE4 inhibitors abrogate the ability of dexamethasone to reduce B-CLL GR $\alpha$  transcript levels.** Exposure to glucocorticoids regulates intracellular GR levels, with resultant down-regulation of GR in most cell lineages, including B cells and B lineage cell lines, but with up-regulation of GR in thymocytes and T-ALL-derived cell lines (28). Using a tetracycline-regulated GR promoter transfected into a cell line lacking functional GR, the glucocorticoid-induced autoinduction of GR expression in human T-cell lines has been linked to their increased sensitivity to glucocorticoid-mediated apoptosis (35). We therefore sought to determine whether in CLL cells, cotreatment with PDE4 inhibitors would abrogate glucocorticoid-mediated attenuation of GR transcript levels. As expected, dexamethasone reduced GR transcript levels in CLL cells in a dose-dependent manner such that following treatment for 6 h with 1  $\mu$ M/L dexamethasone, GR transcript levels were one-third of those observed in untreated cells (Fig. 5B). In contrast, cotreatment of CLL cells for 6 h with 20  $\mu$ M/L rolipram and varying doses of dexamethasone resulted in GR transcript levels above basal levels, even at 1  $\mu$ M/L dexamethasone (Fig. 5B).

These results suggest that PDE4 inhibitors may augment glucocorticoid-mediated apoptosis in B-CLL cells as a result of their ability to block the normal down-regulation of GR transcript levels in glucocorticoid-treated cells.

Given our prior demonstration that inhibition of PKA signaling with the enantiomeric cAMP antagonist Rp-8-Br-cAMPS substantially or entirely blocked the ability of glucocorticoids to induce apoptosis in B-CLL cells as well as reduced transactivation of GRE-containing reporter constructs, we sought to determine whether the same antagonist blocks PDE4 inhibitor-induced augmentation of GR $\alpha$  transcript levels (26). Pretreatment of CLL cells with Rp-8-Br-cAMPS (500  $\mu$ M/L) markedly reduced rolipram-induced augmentation of GR $\alpha$  at 4 h (Fig. 5C). These results are consistent with the hypothesis that PDE4 inhibitors regulate GR $\alpha$  transcript levels by a cAMP- and PKA-mediated mechanism.

## Discussion

This study shows that treatment with several structurally distinct PDE4 inhibitors augments GR transcript levels in B-CLL cells but not in normal circulating hematopoietic cells. Because cotreatment with PDE4 inhibitors and glucocorticoids also

induces apoptosis in B-CLL cells to levels higher than that observed with either agent alone, these results suggest that the simultaneous use of these two classes of drug might be relatively selectively toxic to CLL cells (26). Although it is experimentally difficult to determine whether the alterations in GR expression account for the augmented apoptosis observed when these drugs are combined, a variety of prior studies have shown that levels of GR can play an important role in determining the outcome of glucocorticoid treatment. In cell lines expressing different levels of GR, the magnitude of transcriptional responses to glucocorticoids are roughly proportional to the number of receptors per cell (36). Thymocytes from transgenic mice carrying two extra copies of the GR show enhanced sensitivity to glucocorticoid-mediated apoptosis (37).

Consistent with actinomycin D experiments demonstrating that PDE4 inhibitors do not substantially alter GR transcript half-life, we find that rolipram augments GR transcripts derived from different promoters to varying degrees in CLL cells, suggesting a transcriptional mechanism for the observed increase in GR transcript levels. GR transcription in human lymphoid cells is regulated by at least three promoters (A-C), although the open reading frame of the GR gene, which begins in exon 2, is not altered by promoter usage (32). A quantitative analysis in the IM-9 human B cell line revealed that in such cells, promoters 1A, 1B, and 1C accounted for 1%, 23%, and 76% of all GR $\alpha$  transcripts, respectively (28). Alternative splicing of transcripts derived from the most 5' promoter, 1A, results in three types of transcripts: 1A1, 1A2, and 1A3, the last of which was the most abundant (32). Although a prior study in HeLa cells has suggested cAMP-mediated regulation of the most 3' promoter, 1C, the effects of cAMP signaling on GR promoters 1A and 1B have not been reported (21). Transcripts containing 1B and 1C seem to be relatively ubiquitously expressed, whereas expression of exon 1A3-containing transcripts is particularly high in cell lines of hematopoietic lineage (32). In B-CLL cells, treatment with PDE4 inhibitors augments 1A3 transcripts to a greater degree than other GR transcripts (1A3 > 1B;  $P < 0.05$ ). It is of interest that basal levels of GR transcripts containing exon 1A correlate with sensitivity to glucocorticoid-induced apoptosis because expression of this form of GR transcript is particularly high in thymocytes and T lymphoblastoid cell lines (38). Although the functional significance of the fact that PDE4 inhibitors preferentially induce expression of exon 1A3-containing GR transcripts in B-CLL cells remains unknown, it is possible that varying 5' untranslated regions could alter either GR mRNA translation start site or translation efficiency. Two alternative translation initiation sites in exon 2 give rise to the A and B forms of the GR. GR $\alpha$  B has twice the biological activity of GR $\alpha$  A in gene expression studies, and different tissue types have differing ratios of GR $\alpha$  A and GR $\alpha$  B (39, 40).

Among all the primary hematopoietic cell populations tested, augmentation of GR transcript levels by PDE4 inhibitors is unique to B-CLL cells. This finding is in keeping with a prior study in which, among a variety of circulating hematopoietic

cells examined, PDE4 inhibitor-induced EPAC activation was found only in B-CLL cells (25). The explanation for the unique impact of PDE4 inhibitor treatment on signaling in B-CLL cells remains unknown. Although we do not observe rolipram-induced up-regulation of GR transcript in primary circulating B-cell samples, it is still possible that comparable responses might be observed in a normal B-cell population that is not well represented among circulating B cells. Because PDE4 inhibitors have potent effects on a variety of primary circulating hematopoietic cells, particularly T cells and monocytes, it is clearly not the case that the selective augmentation of GR transcript observed in B-CLL cells is due to the fact that PDE4 inhibitors initiate cAMP-mediated signaling only in B-CLL cells. Instead, the selectivity of the effects observed in CLL cells may be due to the magnitude or kinetics of the cAMP response, the effector proteins activated (i.e., type 1 versus type 2 PKA, EPAC) or, perhaps most likely, cell type-specific signaling induced by cAMP effector protein activation in B-CLL cells.

Glucocorticoids regulate expression of GR. In lymphoid cell subsets that are particularly sensitive to glucocorticoid-mediated apoptosis, glucocorticoids augment GR transcript levels (41). In other cell lineages, including B lineage cells, glucocorticoids reduce GR $\alpha$  transcript levels (28). Consistent with this literature, we find that treatment of B-CLL cells with dexamethasone reduces GR $\alpha$  transcript levels in a dose-dependent manner. In contrast, treatment with the combination of dexamethasone and a PDE4 inhibitor augmented GR $\alpha$  transcript levels. Although treatment with both drugs resulted in the augmentation of GR $\alpha$  transcript intermediate between those observed with treatment with either drug alone, even at the highest dose of dexamethasone used (1  $\mu$ M/L), cotreatment resulted in an increment rather than a decrement in GR $\alpha$  transcript levels. These results suggest that PDE4 inhibitors may increase glucocorticoid-mediated apoptosis in B-CLL cells because they counteract the normal dampening of glucocorticoid-mediated signaling that occurs in B lineage cells as a result of glucocorticoid-induced down-regulation of GR $\alpha$  levels.

High-dose glucocorticoid therapy can lead to clinical responses in a subset of patients with treatment refractory B-CLL (3, 4). The experiments described in this study suggest that the addition of a PDE4 inhibitor to such therapy might fairly selectively augment apoptosis in B-CLL cells as a result of augmentation of GR transcript levels. Our observation that treatment with PDE4 inhibitors for as few as 4 h augments glucocorticoid-induced killing of B-CLL cells augers well for the potential clinical applicability of PDE4 inhibitor/glucocorticoid therapy for B cell malignancies because it is likely that therapeutically effective serum levels of PDE4 inhibitors could be safely maintained for such a period of time. Our future studies will focus on the mechanisms underlying the selectivity with which PDE4 inhibitors alter cAMP metabolism in CLL cells.

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